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FROM NUCLEUS TO MITOCHONDRIA: A UBIQUITINATION STUDY

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Karolinska Institutet

Stockholm 2019

Published by Karolinska Institutet.
Printed by Eprint AB 2019

ISBN 978-91-7831-306-8

From nucleus to mitochondria: a ubiquitination study

THESIS FOR LICENTIATE DEGREE

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In the loving memory of my grand-fathers: Jean Le Doussal and Charles Richard.

It may all have started as a pleasant walk, a careless journey, free from the dreaded expectation that one can feel today.

However, step by step, you went forward, and before even realising it, you started climbing.

Past fellows are now gone. They've taken another path, lost in the fog of possibilities. Only a few remain, that have started climbing on their own.

So look up now, and as you climb one rock after the other, know there is joy in this struggle.

The peak, hidden by a veil of mist, is never to be seen, nor conquered, for it is merely but an illusion.

[...]

As you sweat, bleed and wonder what other paths could have been, you may find what you were looking for. The fulllfillness. The feeling of being exactly where you belong, peace of mind within reach.

Yet, this is another gamble on the lottery of paths. Despite all you have given, all your efforts and all your strength, you might end up finding nothing but bitterness.

So climb, and feel it. And if you look down, remember to smile at your past blood and sweat-stained rocks that stand below, for they have carved what you are.

- Anachronism, Hidden Relief

ABSTRACT

Ubiquitination is a post-translational modification achieved by an enzymatic cascade. This post-translational modification is involved in many crucial cellular processes across different cellular compartments such as protein turnover via the ubiquitin proteasome system and various signaling pathways from the DNA damage response to the immune response. Ubiquitin has seven lysine residues (Lys 6, 11, 27, 29, 33, 48 and 63) onto which other ubiquitin moieties can be conjugated, forming ubiquitin chains of different types. These different ubiquitin chains can have different functions and the relationship between both is often referred to as “the ubiquitin code”. Although the enzymatic cascade leading to ubiquitination of proteins is well described, the ubiquitin code remains largely unresolved. Accumulation of insoluble ubiquitinated proteins is a hallmark of neurodegenerative disorders such as Parkinson’s and Alzheimer’s disease, making the study of these cellular processes relevant to human health. Specific proteins can also impair the function of the proteasome such as progerin in the Hutchinson-Gilford Progeria Syndrome (HGPS).

The importance of ubiquitination in many cellular processes and its involvement in many human pathologies inspired us to develop an inducible ubiquitination system that could be used as a tool to better understand the ubiquitin code and its role in different cellular compartments. In paper I, we have engineered a ubiquitin ligase, ProxE3, which assembles specific ubiquitin chain (lysine 63) onto a fluorescent substrate. We have used this tool to generate ubiquitin chains on the surface of mitochondria and investigate mitophagy, more precisely if lysine 63 (K63) ubiquitin chains are sufficient to trigger aggregation of mitochondria or mitophagy. Upon successful ubiquitination of the surface of mitochondria by ProxE3 and depolarization of mitochondria by carbonyl cyanide m-chlorophenyl hydrazone (CCCP), peri-nuclear clustering of mitochondria was observed but not mitophagy. The lack of mitophagy indicates that either the amount of K63 ubiquitination is insufficient in our system, that other types of ubiquitin chains are required, that a specific substrate need to be ubiquitinated or that the PINK1 feedforward loop is essential for mitophagy. Nonetheless, this work presents a valid tool for studies of ubiquitination in living cells, while reaffirming the complexity of the regulation of mitophagy.

In paper II, we were interested in ubiquitination in a different cellular compartment: the nucleus. In this paper, we used a human cell-line expressing a fluorescent proteasomal substrate to investigate if the ubiquitin-proteasome system was impaired upon overexpression of progerin. Progerin is a mutated form of lamin A which is the cause of HGPS. It has been suspected that progerin might inhibit the catalytic activity of the proteasome, which could lead to neuronal dysfunction. However, we did not detect proteasomal impairment in human cells overexpressing progerin compared to wild-type lamin A. This observation is further supported by the lack of progerin/lamin A inclusions in hippocampal neurons of HGPS mice, implying that the ubiquitin/proteasome system is not sensitive to the expression of progerin in neurons of mice.

LIST OF SCIENTIFIC PAPERS

I. **Thibaud J.C. Richard**, Aldwin Suryo Rahmanto, Olle Sangfelt, Florian A. Salomons and Nico P. Dantuma.
An engineered ubiquitin ligase for inducible K63-linked ubiquitylation.
Manuscript

II. Jean-Ha Baek, Eva Schmidt*, Nikenza Viceconte*, Charlotte Strandgren*, Karin Pernold, **Thibaud J.C. Richard**, Fred W. van Leeuwen, Nico P. Dantuma, Peter Damberg, Kjell Hultenby, Brun Ulfhake, Enrico Mugnaini, Björn Rozell and Maria Eriksson.
Expression of progerin in aging mouse brains reveals structural nuclear abnormalities without detectible significant alterations in gene expression, hippocampal stem cells or behavior.
Hum. Mol. Genet. 2015 Mar;24(5):1305-21.

* These authors contributed equally.

CONTENTS

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AOTF	Acousto-optic tunable filter
ATG	Autophagy related
ATP	Adenosine triphosphate
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CHIP	Carboxyl terminus of Hsc70-Interacting Protein
DNA	Deoxyribonucleic Acid
DUB	Deubiquitinating enzyme
E1	Ubiquitin activase
E2	Ubiquitin conjugase
E3	Ubiquitin ligase
E6AP	E6 Associated Protein
ECL	Enhanced chemiluminescence
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum-Associated Degradation
FANCD2	Fanconi Anemia Complementation Group 2
FKBP12	12-kDa FK506 Binding Protein
FRB	FKBP-Rapamycin Binding domain
GFP	Green Fluorescent Protein
Gly	Glycine
HECT	Homologous to E6AP C-terminus
HeLa cells	Henrietta Lacks cells
HDAC6	Histone deacetylase 6
HGPS	Hutchinson-Gilford Progeria Syndrome
HIF1α	Hypoxia-Inducible Factor-1 alpha
HRP	Horseradish Peroxidase

IBMPFD	Inclusion Body Myopathy associated with Paget's disease of the bone and Fronto-temporal Dementia
IBR	In-between RING domain
IMM	Inner Mitochondrial Membrane
JAMM	JAB1/MPN/Mob34 metalloenzyme
LIR	LC3-Interacting Region
LMNA	Lamin A
Lys/K	Lysine
MET	Methionine
MFN	Mitofusin
MHC	Major Histocompatibility Complex
MPP	Mitochondrial Processing Peptidase
MTS	Mitochondrial Targeting Signal
NEDD	Neural precursor cell Expressed Developmentally Down-regulated
OMM	Outer Mitochondrial Membrane
OPTN	Optineurin
OTU	Otubain protease
PBS	Phosphate-buffer saline
PE	Phosphatidylethanolamine
PI(3)P	Phosphatidylinositol 3-phosphate
PINK1	PTEN-induced putative kinase 1
PMT	Photomutiplier tube
PVDF	Polyvinylidene difluoride
PROTAC	Proteolysis targeting chimeras
PTEN	Phosphatase and tensin homolog
RBR	RING-in-between-RING
RHOT1	Ras homolog family member T1
RING	Really Interesting New Gene
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SQSTM1	Sequestosome 1
TBS	Tris-Buffered Saline
TIM	Translocase of the Inner Membrane
TOM	Translocase of the Outer Membrane
UBA	Ubiquitin-associated domain
UBD	Ubiquitin Binding Domain
UBL	Ubiquitin-like domain
UCH	Ubiquitin C-terminal Hydrolase
UIM	Ubiquitin Interacting Motif
UPS	Ubiquitin-Proteasome System
USP	Ubiquitin-Specific Protease
VHL	Von Hippel-Lindau

1 INTRODUCTION

A cell is able to respond to its environment via a plethora of modifications of its genome and proteome, for instance translation regulations by DNA methylation and protein post-translational modifications. Post-translational modifications are diverse and can change proteins structure and functions chemically (phosphorylation, acetylation, methylation, S-nitrosylation, thiolation, S-sulfonation, glycosylation, acylation, prenylation, AMPylation, ADP-ribosylation and S-sulfenylation) or *via* polypeptides (ubiquitination, ubiquitin-like, neddylation and SUMOylation) [1]. The primary focus of my thesis is ubiquitination and its involvement in the fate of proteins in different cellular compartments. Other post-translational modifications are beyond the scope of this work, unless they are involved in a relevant cross-talk with ubiquitination, such as acetylation, phosphorylation [2,3] and SUMOylation [4].

1.1 UBIQUITIN

The ubiquitin is a small 76 amino acid-long protein (see **Fig. 1**), which was first characterized for its role in post-translational modification of histones [5,6] but also ubiquitin-dependent proteasome degradation [7] and subsequently, regulation of gene expression [8–11] and DNA damage response [12,13]. Ubiquitination has been further shown to be a major post-translational modification involved in most cellular processes such as apoptosis [14], the cell-cycle, endocytosis, inter-cellular communication [15,16], macroautophagy (referred to as autophagy) and mitophagy. The role of ubiquitination in autophagy and mitophagy will be further discussed in this introduction.

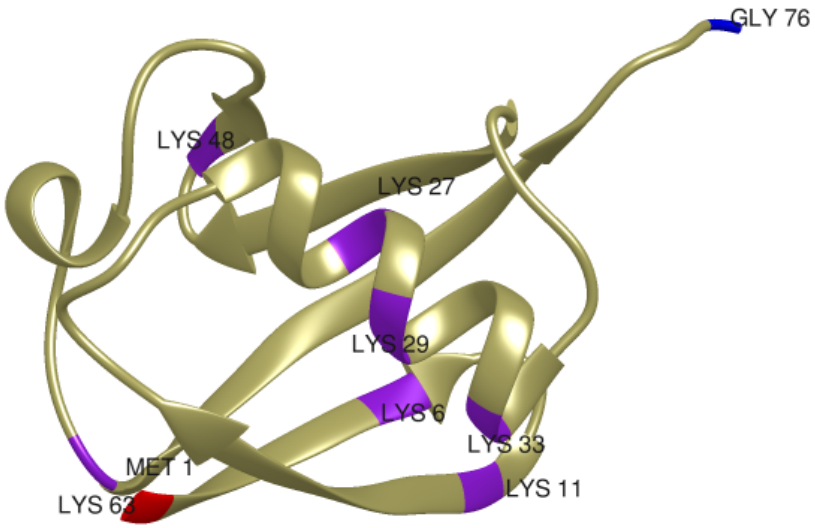

FIGURE 1. Structure of ubiquitin. Image of 1UBQ [17] created with UCSF Chimera [18]. The N-terminal methionine (MET) is colored in red, while the C-terminal glycine (Gly) is colored in dark blue and lysine (Lys) residues in purple.

1.2 UBIQUITINATION

The cellular process known as ubiquitination is a reversible post-translational modification driven by an enzymatic cascade resulting in ubiquitin being conjugated onto a specific lysine residue of a substrate [19] (see **Fig. 2**). At the beginning of this enzymatic cascade [7], the ubiquitin activase (E1) acetylates the ubiquitin in an ATP dependent manner. After this step, the activated ubiquitin will be transferred via a trans(thio)esterification reaction from the E1 onto a ubiquitin conjugase (E2). Finally, a ubiquitin ligase (E3) specific for a substrate will facilitate the conjugation of the ubiquitin by the E2 onto the substrate, although some E3 ligases have themselves an intrinsic catalytic activity that directly mediates the transfer of the ubiquitin. This final step results in the formation of an isopeptide bond between a lysine of the substrate and the C-terminal glycine of ubiquitin. The final conjugation step of a ubiquitin moiety unto a substrate varies depending on the type of E3, as discussed later in this introduction. A ubiquitin moiety can be conjugated onto another ubiquitin moiety's lysine residues K6, 11, 27, 29, 33, 48 and 63 (see **Fig. 1**). Sequential conjugation events result in the formation of ubiquitin chains. While hundreds of these E3 are known to exist [20], only two E1 (UBA1/UBE1 and UBA6) [21,22] and about forty E2s have been found [23], making E3 ligases attractive therapeutic targets in a wide range of diseases due to their diversity and target specificity [24–26].

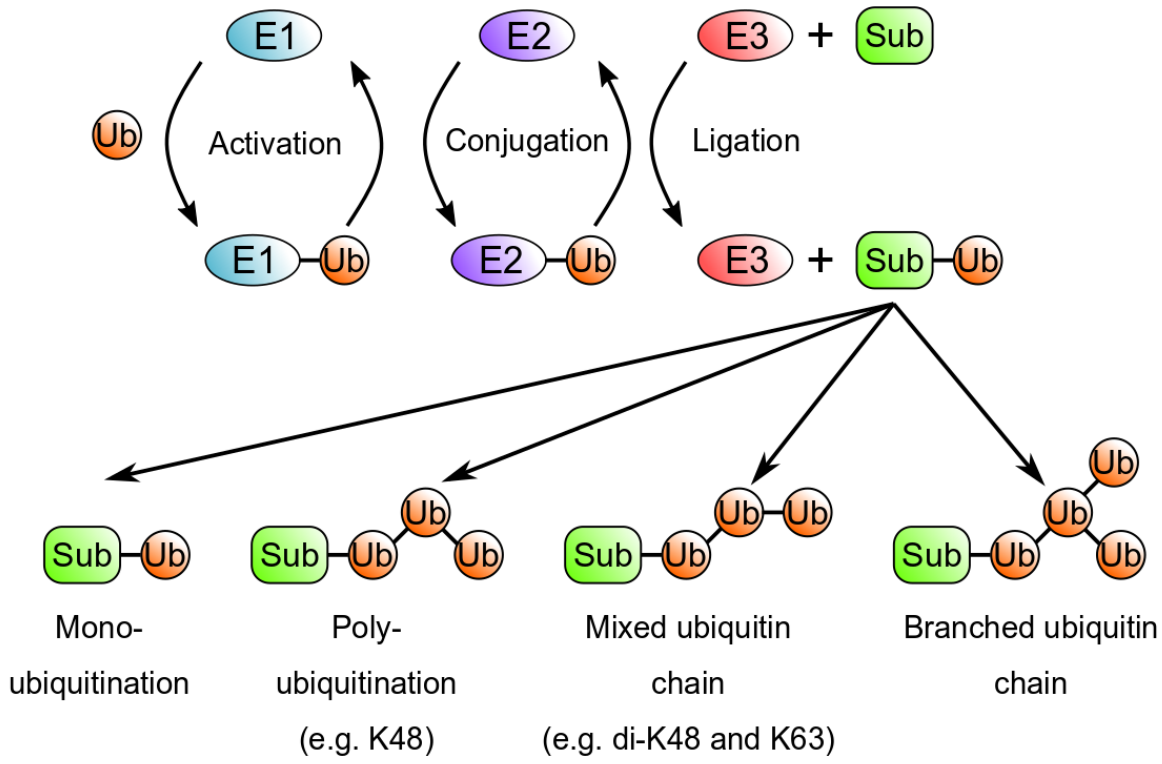

FIGURE 2. Ubiquitination process and resulting types of ubiquitination. Ubiquitin activase (E1) is colored in blue, ubiquitin conjugase (E2) is colored in purple, ubiquitin ligase (E3) is colored in red, ubiquitin (Ub) is colored in orange and the substrate (Sub) is colored in green.

1.3 UBIQUITIN LIGASES

Three main families of E3s are known so far. The Really Interesting New Gene (RING) [27,28] the Homologous to E6AP C-terminus (HECT) [29–31] and the RING-in-between-RING (RBR) [32–34] family (see **Fig. 3**). The RING E3 facilitates the conjugation of ubiquitin as described previously. It functions as a scaffold protein, bringing the E2 in a closed proximity with the substrate [23], thus facilitating the transfer of the ubiquitin moiety from the E2 to the substrate. RING E3s therefore lack a catalytic domain. Unlike the RING family, the HECT and RBR families possess an intrinsic catalytic activity. In the case of HECT E3, the catalytic cysteine in the HECT domain forms a thioester bond with ubiquitin before catalyzing the formation of the isopeptide bond between the ubiquitin and the lysine residue in the substrate. On top of their HECT domain, most E3 of this family have up to four WW domains and a C2 domain involved in protein/protein interactions, including substrate recognition. Since HECT E3s have an intrinsic catalytic activity, they are able to auto-ubiquitinate as a regulatory mechanism if intra-molecular interactions between the HECT domain and a WW domain or C2 domain are disrupted, leading to their own degradation [35–37]. HECT ligases also have an inactive closed conformation when their WW or C2 domains are interacting with their HECT domain, thus reducing the accessibility of the substrate to the WW domains or the E2 to the HECT domain. Such interaction can be intra-molecular [38] or inter-molecular in the case of the NEDD4 family member Smurf1, which forms inactive dimers [39]. It is also worth mentioning that more evidence is pointing towards sequential formation of ubiquitin chains by HECT E3 as they transfer one ubiquitin moiety at a time from the E3~Ub to the substrate [40–42]. The sequential-addition model of ubiquitin supports chain elongation and formation of branched ubiquitin chains [43]. This sequential formation of ubiquitin chains is in opposition with the *en bloc* ligation of a chain. During this process, a chain is first assembled on the E2, then transferred to the active cysteine of an E3 and finally transferred to the substrate [44]. The chain specificity of HECT E3s is purely dependent on their HECT domain and not the E2 that donates the ubiquitin to their catalytic site [40]. Mechanistically, RBR presents features of RING and HECT E3s. This family of E3s have two RING domains (RING 1 and RING 2) separated by an “in-between RING domain” (IBR). RING 1 is involved in the binding of E2~ubiquitin while RING 2 is the catalytic domain involved in the transfer of ubiquitin on the substrate, while the IBR is helping both of these functions.

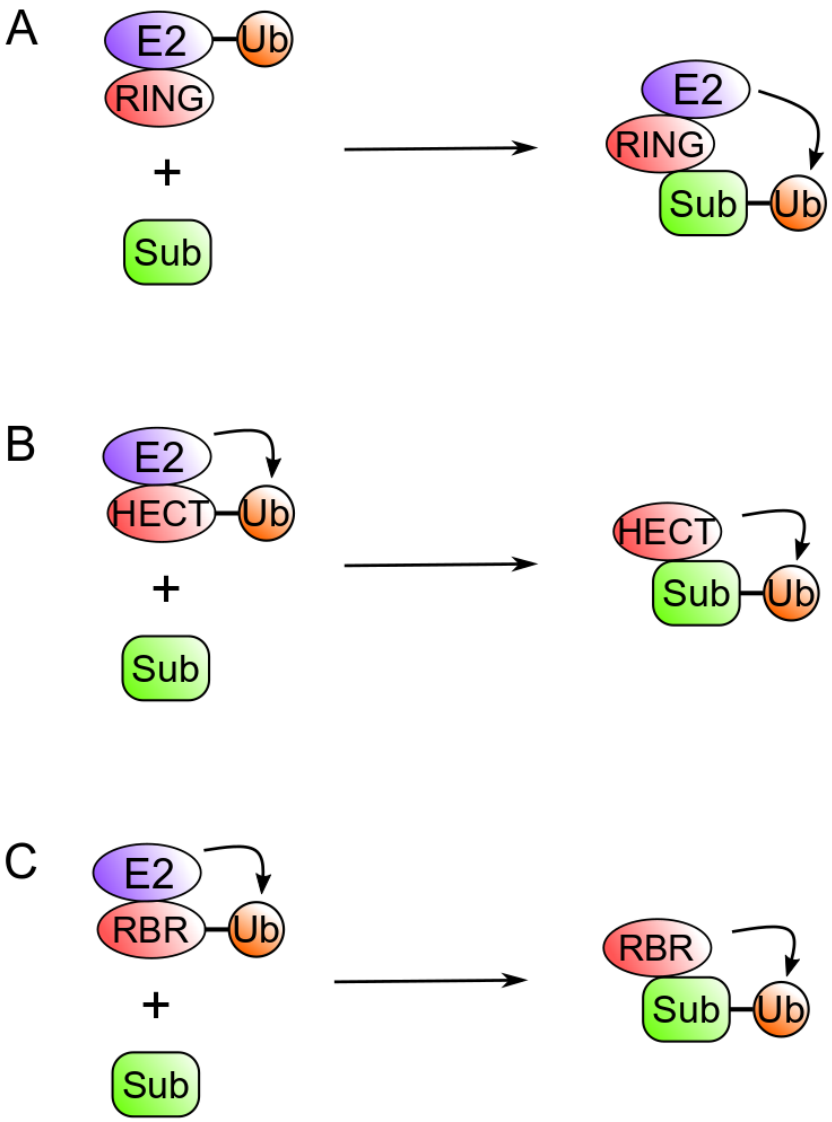

FIGURE 3. Main families of ubiquitin ligases. Simplified representation of ubiquitination of a substrate by A) RING ubiquitin ligases B) HECT ubiquitin ligases and C) RBR ubiquitin ligases. Ubiquitin conjugase (E2) is colored in purple, ubiquitin ligases (E3) are colored in red, ubiquitin (Ub) is colored in orange and the substrate (Sub) is colored in green. Bent arrows represent the transfer of ubiquitin.

1.4 UBIQUITIN CHAIN TYPES

A single ubiquitin moiety can be conjugated onto a substrate (see **Fig. 2**), which is referred to as monoubiquitination. Ubiquitin moieties can also be linked to each other via their lysine residues (K6, K11, K27, K29, K33, K48 and K63) or their N-terminal end (M1-linked ubiquitination also known as linear ubiquitin chains) to form ubiquitin chains. This is known as polyubiquitination. These ubiquitin chains can be homogenous, mixed or branched with different types of ubiquitin chains having different functions, resulting in the "ubiquitin code" [45]. Homogenous chains are made of ubiquitin conjugated to each other via the same linkage. Depending on the linkage, a polyubiquitin chain will have a different structure. For example, K48 linked chain are more compact than K63 chains, which are very similar to linear ubiquitin chains. With this example in mind, and since structure influences function, K48 homogenous ubiquitin chains are known to target for protein degradation via the proteasome [46], while K63 homogenous chains have been traditionally shown not to be involved in proteasomal degradation [47] but fulfill non-proteolytic roles, such as the DNA damage response [48] or endocytosis [49,50]. However, the distinction between proteolytic and non-proteolytic ubiquitin chains becomes less clear as progress towards understanding of the ubiquitin code is made. It has for example been shown that not only K48 linkages but also K6, K11, K27, K29 and K33 chains are increased in cells upon inhibition of the proteasome [47]. Furthermore, K48 ubiquitin chains can have non-proteolytic roles in regulation of transcription factors [51], while K63 chains can target proteins for lysosomal degradation [52] and K63/K48 mixed chains can target for proteasomal degradation [53]. These mixed ubiquitin chains consist of a mix of ubiquitin linkages within a chain and can even branch out. Recently, branched ubiquitin chains have been shown to be assembled in two phases by the HECT E3 WWP1. First a unidirectional K63 ubiquitin chain is assembled on the substrate and in a second step, mixed branches are added to the ubiquitin chain [43]. Mixed and branched ubiquitin chains render the understanding of the ubiquitin code even more challenging, as it complicates the ubiquitination landscape considerably. For example, while homogeneous K11 ubiquitin chains do not target proteins involved in the cell-cycle for degradation by the proteasome, mixed K11/K48 ubiquitin chains do [54,55]. To complicate things even further, mixed chains have been shown to have different conformations depending on the position of a given linkage in a tri-ubiquitin chain (see **Fig. 2**, mixed ubiquitin chain), which affects the cleavage rate of specific ubiquitin linkages by deubiquitinating enzymes [56].

1.5 REGULATION OF UBIQUITINATION AND DEUBIQUITINATING ENZYMES

Ubiquitination regulates many cellular processes, as discussed previously, and it is therefore a reversible and tightly regulated process itself. HECT E3 activity is regulated by inter- or intra-molecular interactions that keep the HECT E3 in an inactive conformation and also prevents auto-ubiquitination. Substrate adaptor subunits of E3 ligases, such as Cdh1 (from the multisubunit E3 APC/C), have also been shown to be able to lift inhibitory inter-molecular interactions to activate other E3s [39] or to inhibit E3s by facilitating inhibitory intra-molecular interactions [57], both independently of APC/E3 activity. Other ways of regulating ubiquitination of substrates are through deubiquitinating enzymes (DUBs). These proteases target ubiquitin conjugates and ubiquitin chains and reverse the ubiquitination modification. Most DUBs are cysteine proteases and belong to five different families based on their catalytic domains: ubiquitin C-terminal hydrolase (UCH), ubiquitin-specific protease (USP), Otubain protease (OTU) and Josephin domain DUBs [58]. A smaller number of DUBs are metalloproteases and belong to the JAB1/MPN/Mob34 metalloenzyme (JAMM) family. The substrate specificity of DUBs can depend on a given type of ubiquitin chain - for example the yeast DUB Ubp2 cleaves K48 ubiquitin chains rather than K63 [59] - while mammalian USP14 cleaves K48 ubiquitin chains but not K63 [60]. However, DUBs can also be specific for a certain ubiquitinated target. For example, the DUB USP8 deubiquitinates and stabilizes the RING E3 ligase NRDP1. NRDP1 is known to auto-ubiquitinate leading to its own degradation in absence of USP8 [61]. Ultimately, the specificity of a DUB depends on both the ubiquitin chains and the ubiquitin-modified target. This is, for example, illustrated by USP8, which specifically removes K6 ubiquitin chains conjugated on the RBR E3 PARKIN [62,63], which is involved in mitophagy. Interactions between DUBs and E3 paint a complex picture of ubiquitination as this can result in situation where ubiquitin chains are not only conjugated or cleaved, but instead edited with one linkage being replaced for another linkage [64,65].

The binding of DUBs to ubiquitin is possible through interaction between the ubiquitin and the ubiquitin binding domain (UBD) of a DUB. UBDs are extremely diverse: about twenty different UBDs domains have been described so far [66]. These UBDs subclasses can be of various structure such as alpha-helical, Zinc finger, Plekstrin-like, Src homology 3 or WD40 beta-propeller. UBDs are found not only in DUBs but also in many other proteins involved in ubiquitin-dependent pathways. Beside their well-established function in the NF-κB pathway [67] and DNA damage response [68], UBDs are found in molecular chaperones such as p97/VCP (Cdc48 in yeast) cofactors Ufd1, Npl4 and p47 [69,70], which are involved in membrane fusion, endoplasmic reticulum (ER)-associated degradation (ERAD), export of misfolded proteins from the ER and proteasomal degradation [71,72]. UBDs are also found in

proteins involved in endocytosis such as epsins, EPS15 and EPS15R [73,74]. In addition, autophagic adaptors such as p62/SQSTM1 [75] and NBR1 [76] possess UBDs, allowing for autophagic degradation of ubiquitinated substrates. Finally, shuttle factors involved proteasomal degradation and intrinsic ubiquitin receptors of the proteasome contain UBDs.

1.6 UBIQUITIN IN THE NUCLEUS

Complementarily to its nuclear functions in DNA damage response, histone modifications and gene regulation, as mentioned earlier in this introduction, ubiquitin can regulate subcellular localization, including nuclear localization of proteins, through monoubiquitination [77]. For example, the Fanconi Anemia Complementation Group 2 (FANCD2) protein, involved in DNA damage response, requires monoubiquitination on it lysine residue 561 in order to localize to nuclear foci in response to DNA damage. The UPS has also been described to be enriched in the nucleus of specific cell types [78], such as certain populations of pyramidal neurons and cortical neurons in rats [79]. The nuclear localization of proteasomes in mammalian cells has been shown to occur both rapidly during mitosis and slowly by diffusion through the nuclear envelope [80]. Nuclear proteasomes have been shown to be important for degradation of transcription factors, proteins from the nuclear envelope and misfolded proteins [81–83]. Components of the nuclear envelope such as lamin A have also been shown to be degraded by another proteolytic process, which occasionally relies on ubiquitin: autophagy [84]. Mutations in lamin A – the mutant protein being referred to as progerin – are responsible for the Hutchinson-Gilford progeria syndrome (HGPS) [85]. In the context of HGPS, inducing autophagy via rapamycin treatment [86] has been shown to alleviates phenotypes [85,87]. Similarly, targeting autophagy or the UPS in neurodegenerative disorders such as Parkinson's, Alzheimer's, Huntington's disease and aging in general, which are all conditions where misfolded proteins accumulate in the nucleus of neurons, has been proposed as a therapeutic approach worth investigating [88–90]. In short, by harnessing ubiquitin signaling, it may be possible to induce degradation of proteins involved in pathologies [91]. Although this idea is not new, it did not translate into therapies used in a clinical setting so far. This is probably partially due to the complexity of the ubiquitin code and the large amount of time necessary to develop the molecular tools required for understanding it.

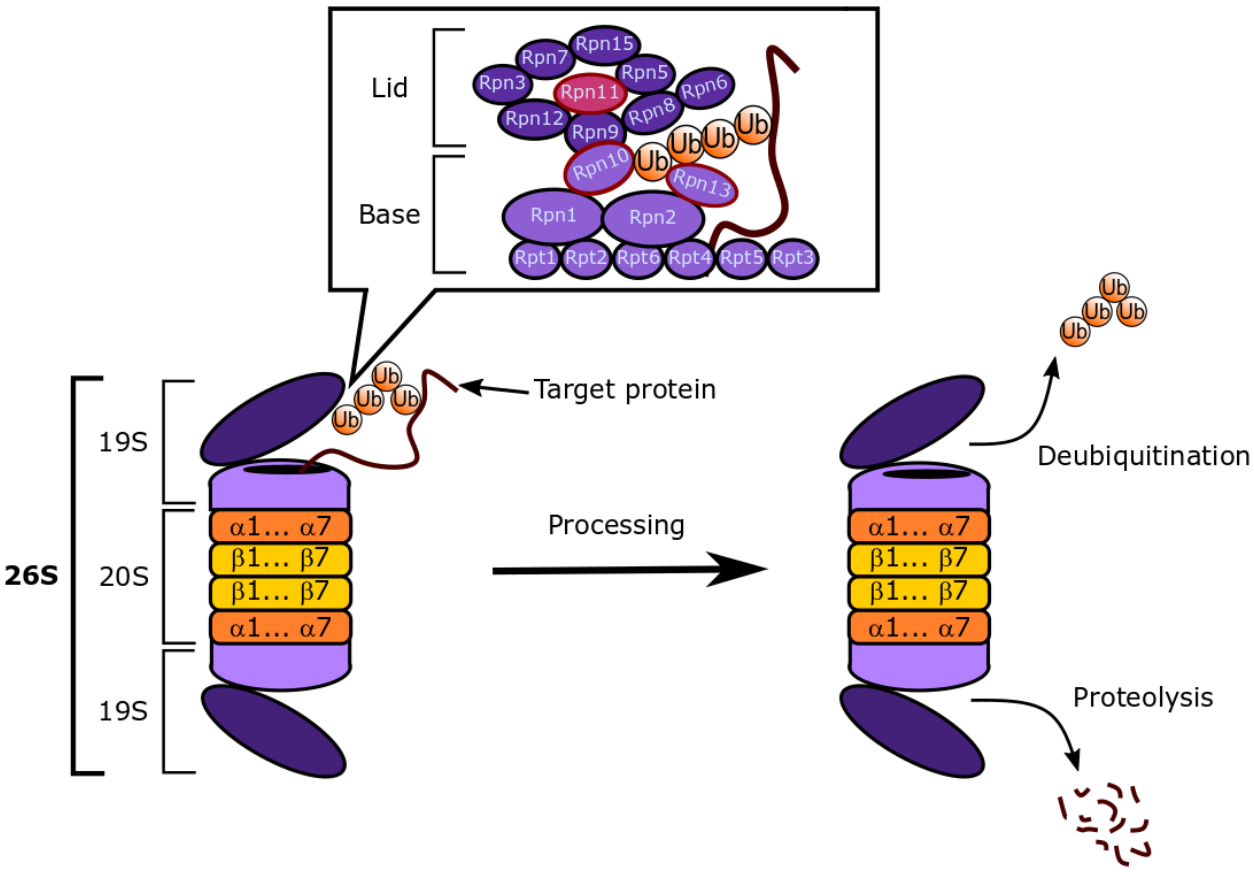

FIGURE 4. Simplified 26S yeast proteasome structure and proteasomal degradation of a protein. Subunits interacting with ubiquitin (Rpn10 and Rpn13) are represented with a dark red border. Rpn11, which deubiquitinates the target protein, is colored in pink. The lid of the 19S regulatory particle is colored in dark purple. The base of the lid is colored in lilac. The alpha ring is colored in orange. The beta ring is colored in yellow.

1.7 UBIQUITIN PROTEASOME SYSTEM

1.7.1 Structure of the proteasome

The mammalian 26S proteasome is a cylindrical 2.5 MDa protein complex, composed of a 20S central core particle and capped by two 19S regulatory particles (see **Fig. 4**). This protein complex is made of 33 subunits. The core particle is composed of four rings: two β rings forming the center of the hollow cylinder, each flanked by an α ring. Each ring constitutes of seven α or β subunits. Proteolytic function is achieved by three β subunits in each beta ring: β1, β2 and β5, which have a caspase-like, trypsin-like and chymotrypsin-like activity, respectively. These different activities allow for rapid proteolysis of almost any protein into polypeptides of 7 to 8 amino-acids on average [92].

1.7.2 Substrate recognition

1.7.2.1 Proteasomal subunits

Proteasomal substrate recognition is mediated by different proteins: three proteasomal subunits and several shuttling factors containing Ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains. The proteasomal subunits RPN1, PSMD4 and ADRM1 in mammals (Rpn1, Rpn10 and Rpn13 in yeast, respectively) are part of the 19S regulatory particle, but only Rpn1 seems essential for growth in yeast [93]. Recognition of substrate by RPN1 is mediated by the receptor site T1 in the first toroidal domain of RPN1, which binds preferably K6 and K48-linked diubiquitin and the UBL domain of RAD23, a shuttle factor involved in targeting of ubiquitinated substrates. These interactions have been shown both in *S. cerevisiae* and humans [94]. PSMD4/Rpn10, was the first proteasomal ubiquitin receptor identified [95]. Ubiquitin recognition is mediated by the UIM (an alpha-helical UBD) of Rpn10 and modulated by ubiquitination of Rpn10 itself. In yeast, monoubiquitination of Rpn10 by Rsp5 (a NEDD4-like E3) reduces the affinity of Rpn10 for ubiquitin and ubiquitinated substrates [96]. Deubiquitination of monoubiquitinated Rpn10 by the DUB Ubp2 counters the effects of Rsp5. ADRM1/Rpn13 recognizes monoubiquitin and K48-linked diubiquitin through its N-terminal plekstrin-like domain, which also binds RPN2, anchoring ADRM1/Rpn13 to the 19S particle [97]. The C-terminal half of human ADRM1 binds and activates the DUB UCH37/UCHL5 by lifting its autoinhibition [98,99], effectively recruiting it to the proteasome in order to trim ubiquitin chains from the ubiquitinated substrates targeted for degradation [100], thereby recycling ubiquitin and maintaining the cellular free ubiquitin pool, like PSDM14/POH1/Rpn11 [101] and Ubp6 [60,102,103]. Similarly to PSMD4/Rpn10, ADRM1/Rpn13 interacts with

shuttling factors with UBL and UBA domains such as RAD23, DSK2 [97] and PLIC-2 [94]. Similarities with PSMD4/Rpn10 do not end there, as the affinity for ubiquitin and ubiquitin substrates of ADRM1/Rpn13 can also be modulated by the HECT E3 UBE3C/HUL5. This E3 can polyubiquitinate PSMD4/Rpn10, resulting in decreased binding of ubiquitinated substrates to the proteasome [104].

1.7.2.2 Shuttling factors

UBL/UBA shuttling factors that are not directly bound to the proteasome (unlike Rpn1, Rpn10 and Rpn13) can exist in an auto-inhibited *cis* or *trans* state. Three shuttle factors are known in yeast: Rad23, Dsk2 and Ddi1. The human genome encodes several other shuttling factors [105], namely: HR23A and HR23B (both homologues to Rad23 in yeast), DDI1 and DDI2 [106] (both homologues to Ddi1 in yeast), PLIC-1, PLIC-2 (homologues to Dsk2 in yeast), A1Up, KPC2, NUB1, NUB1L and p62. These shuttling factors act as adaptors presenting a protein for proteolysis while escaping proteolysis themselves [107,108]. They bind a ubiquitinated substrate with their UBA domain and the proteasome with their UBL domain. After being shuttled to the 19S regulatory subunit, proteins targeted for proteasomal degradation are deubiquitinated by proteasome-associated DUBs and simultaneously translocated from the base of the 19S regulatory particle towards the 20S core particle in an ATP-dependent process driven by an AAA-ATPase hetero-hexameric ring composed of Rpt1 to 6 in *S. cerevisiae* and PSMC 1 to 6 in humans [109]. The proteasomal substrate is then hydrolyzed by the 20S core particle as the AAA-ATPase ring progressively feeds the protein into the proteolytic chamber of the 20S core particle.

1.7.3 Alternative forms of proteasome

It is worth mentioning that several types of proteasome have been described harboring different types of regulatory particles beside the canonical 19S. For example, the 19S regulatory particle of the 26S proteasome can be replaced by an 11S particle [110] composed of proteins belonging to the REG family: the REGα/β and REGγ subunits [111,112]. A 19S particle associated with these REG subunits, thus forms a REG proteasome. The REGγ proteasome for example, is involved in immunity and cancer progression [113,114]. Alternatively, a less known and characterized proteasome can be formed with PA200 as its regulatory particle [115]. 20S core particles lacking the 19S regulatory particle have also been described in degradation of proteins damaged by oxidation stress [116] and proteins with large unstructured regions [117]. Other types of proteasomes can have a different composition of 20S core particle. For example, the immunoproteasome harbors three different β subunits in

its 20S core particle, their expression being induced by IFN-γ: β1i, β2i and β5i [118,119]. These β subunits allow for degradation of the substrate protein into oligopeptides of 8-10 amino-acids which can then be presented as antigens by major histocompatibility complex (MHC) class 1 molecules [120]. Although the immunoproteasome is not essential for antigen presentation and the regular 26S proteasome plays a similar role in antigen presentation, the immunoproteasome has been shown to be critical for the generation of specific cytotoxic T lymphocytes epitopes [121]. Some of these alternative forms of proteasome have also been shown to degrade proteins in a ubiquitin-independent fashion. This is the case for PA200 containing proteasomes involved in acetylated histone degradation [122,123], REGγ proteasomes degrading the cyclin-dependent kinase inhibitor p21 [124] and steroid receptor SRC-3/AIB1 [125] and free 20S proteasome degrading oxidized proteins [116].

1.8 UBIQUITIN IN AUTOPHAGY

1.8.1 Core principles of autophagy

1.8.1.1 Cross-talk between UPS and autophagy

Beside proteasomal degradation, cells have an alternative route for protein degradation, namely autophagy, a tightly regulated and evolutionary well conserved process in which more than 30 autophagy-related (ATG) proteins are involved [126]. Proteins are only one type of macromolecules being degraded by autophagy, as autophagy engulfs part of the cytoplasm and organelles for bulk degradation into the lysosome. This bulk degradation is key to cellular homeostasis and recycling of cellular components (proteins, lipids, organelles and carbohydrates). However, autophagy is not limited to passive engulfment of cytoplasm and organelles. It can be selective by targeting specific substrates for degradation via autophagy receptors, both in a ubiquitin-dependent and -independent fashion [127]. Although autophagy and the UPS are presented in two different parts of this introduction, these two processes are not strictly separated in cells and have been shown to occasionally intersect. The histone deacetylase 6 (HDAC6), for example, has been identified as a compensatory link between autophagy and UPS when the proteasome is impaired in *D. melanogaster* [128]. In this situation, HDAC6 is involved in the autophagic degradation of aggregation-prone proteins. HDAC6 has also been shown to act as an adaptor between the dynein motor complex and K63 polyubiquitinated proteins in mammals, guiding these proteins along microtubules towards the microtubule organizing center, an area where autophagy is very active (rich in autolysosomes) [129]. Other chaperones and co-chaperones containing UBA/UBL, such as BCL-2-associated athanogene 1 and 3 (BAG1 and BAG3), two HSC/HSP70 co-chaperones, have been shown to be a switch between proteasomal and autophagic degradation [130]. The work of Gamerdinger *et al.* indicates that BAG3 forms a complex with p62/SQSTM1, which promotes degradation of polyubiquitinated proteins via autophagy, especially in aging cells [130]. On the other hand, cells expressing mostly BAG1 are degrading the vast majority of their polyubiquitinated proteins via the proteasome route. Another chaperone-like protein, p97/VCP, which is involved in proteasomal degradation as mentioned earlier, as well as in autophagy. In fact, mutations of p97/VCP lead to inclusion body myopathy associated with Paget's disease of the bone and frontotemporal dementia (IBMPFD), an autosomal dominant disease in which accumulation of autophagic structures are observed in patients. The UPS, however, remains functional in cells expressing mutant p97/VCP responsible for IBMPFD [131].

1.8.1.2 The steps of autophagy

The first step of autophagy is the *initiation*, followed by *vesicle nucleation*, *elongation*, *fusion* and finally *degradation*. The *initiation* begins with metabolic stimuli such as nutrients deprivation [132]. Alternatively, rapamycin treatment can also initiate autophagy [86]. Both types of stimuli inhibit the mTORC1 complex (composed among others of mTOR and Raptor). This inhibition then activates the serine-threonine kinase ULK1, which is no longer phosphorylated by the mTORC1 complex. The ULK1 complex (formed of ULK1, ATG13, ATG101 and FIP200) is then activated by phosphorylation of ATG13 and FIP200 by ULK1 [133], which leads to the recruitment of the Beclin-1-Vps34 complex. The *vesicle nucleation* step then starts as the Beclin-1-Vps34 complex localizes at the formation site of the phagophore, a cup-shaped membrane (see **Fig. 5**). It is during this step that a component of the Beclin-1-Vps34 complex, Ambra1 (normally phosphorylated and inhibited by mTORC1 under non-autophagic conditions), recruits the RING E3 TRAF6 which participates in ULK1 K63 ubiquitination [134]. K63 ubiquitination of ULK1 induces its self-association and further stabilization, which in turn leads to Beclin-1 phosphorylation. Beclin-1 phosphorylation enhance the activity of the ATG14L-Beclin-1-Vps34-Vps15 complex [135], allowing Vps34 to produce phosphatidylinositol 3-phosphate (PI(3)P). This increase of PI(3)P leads to recruitment of effector proteins such as DFCP1 [136], WIPI1 [137], WIPI2 [138] and subsequent formation of the omegasome [136]. Next the *vesicle elongation* is driven by ATG proteins in a process reminiscent to ubiquitination: ATG12-ATG5-ATG16L on one hand and LC3/Atg8 on the other hand. ATG12 is activated by the E1-like ATG7, then transferred onto the E2-like ATG10 and finally conjugated to a lysine residue of ATG5. The ATG12-ATG5 then binds ATG16L. Running parallel to this process, LC3-I (Atg8 in yeast) is conjugated to phosphatidylethanolamine (PE), forming the lipid bound LC3-II. This conjugation process is catalyzed by ATG7 (E1-like) and ATG3 (E2-like). While the omegasome is elongating, engulfment of target proteins and organelles is facilitated by autophagy receptors such as p62/SQSTM1 [139], NBR1 [76], optineurin (OPTN) [140], NDP52 [141], and Alfy [142]. These autophagy receptors can recognize ubiquitinated substrates and target them for autophagy, *i.e.*: K63 polyubiquitinated substrates can be recognized by p62 and OPTN [143]. However, autophagy receptors are also mediating ubiquitin-independent recognition of autophagy substrates. For example, although OPTN is specific of K63 polyubiquitinated substrates, it has also been shown to recognize protein aggregates with its C-terminal coiled-coil domain, thus playing a role in ubiquitin-independent clearance of aggregated proteins via autophagy [144]. During the final step of autophagy, the omegasome closes, forming an autophagosome, which will then *fuse* with the lysosome, forming the autolysosome (in mammals) in which engulfed cellular components are degraded. The *fusion* step is different for yeast, however.

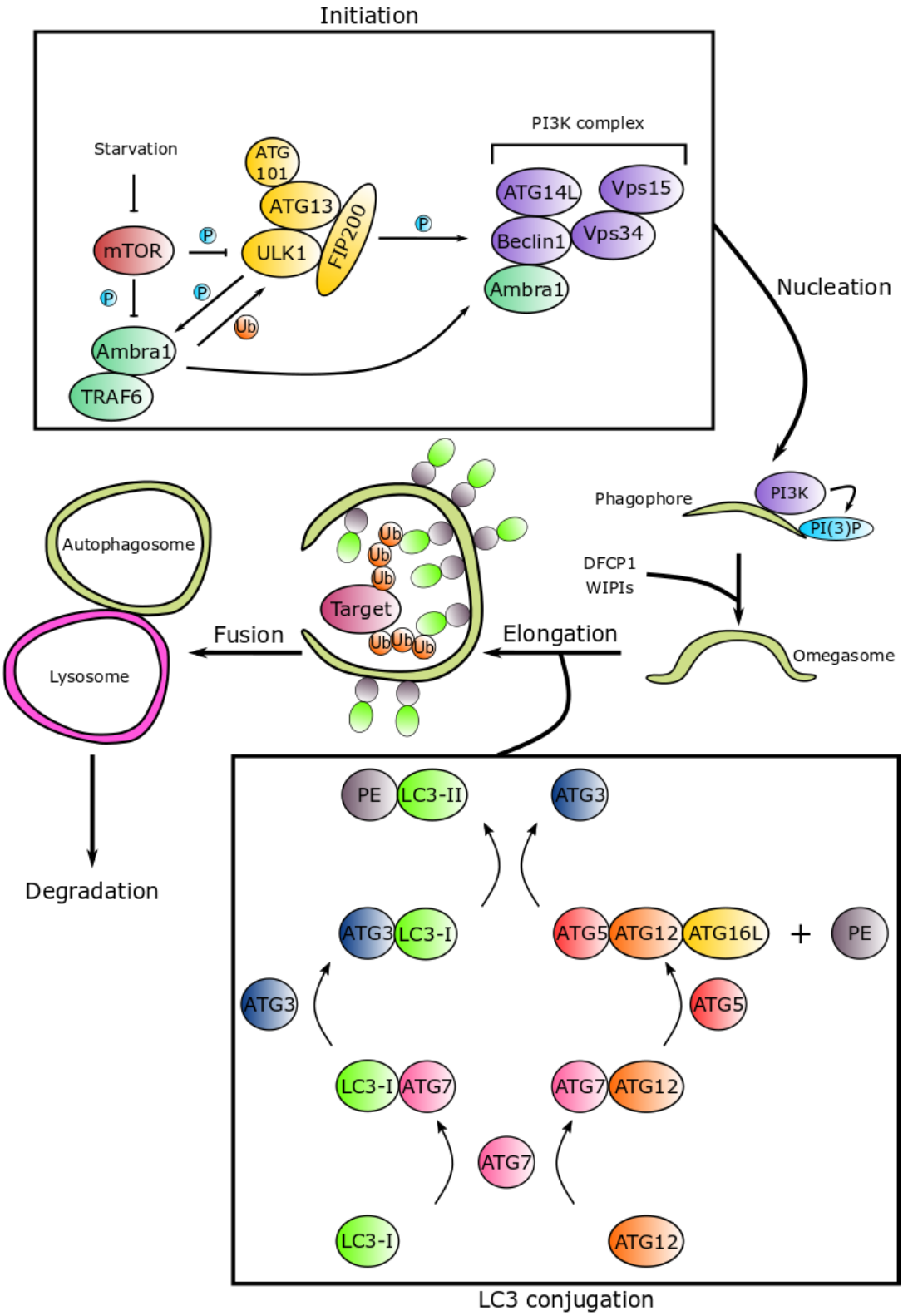

FIGURE 5. Autophagy steps from initiation to fusion and degradation. Inhibiting and activating phosphorylation are represented as a blue circle with the letter “P” inside. K63 ubiquitination is represented as an orange circle with “Ub” written in it.

Autolysosome [145]. It is worth mentioning that the source of the constitutive membrane of omegasomes and autophagosomes are debated to this day [146], although the ER [147], the Golgi apparatus, the plasma membrane and mitochondria [148] are suspected to participate in autophagosome formation.

1.8.2 Mitophagy

1.8.2.1 Mitophagy: a specific autophagy pathway

The previous part of this introduction discussed core principles of autophagy, but several types of autophagy exist depending on the target. Some of these specific autophagy pathways are dependent on Ras-related proteins RAB9A and RAB9B but do not rely on ATG5 and ATG7 unlike conventional autophagy [149–151]. Mitophagy is one of these specific autophagic pathways. It is a degradation pathway dedicated to recycling components of damaged mitochondria in a ubiquitin-dependent and -independent manner [127] and prevents oxidative stress generated by damaged mitochondria and cell death [152]. Various types of stresses can induce mitophagy such as hypoxia [153], starvation [154] or proteotoxic stress in the mitochondrial matrix [155]. Mitophagy has been primarily studied in a stress- or disease-related context, however, recent work using transgenic mice expressing the fluorescent reporter mt-Keima or *mito*-QC have shown that a basal level of mitophagy occurs and vary widely between organs and cell types [156,157]. As mentioned earlier, the RBR E3 PARKIN is involved in mitophagy, although PARKIN-independent forms of mitophagy have been described.

1.8.2.2 PARKIN-dependent mitophagy

PARKIN is activated during ubiquitin-dependent mitophagy by the PTEN-induced putative kinase 1 (PINK1). In healthy mitochondria, PINK1 is transported from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) thanks to its N-terminal mitochondrial targeting signal (MTS) via the translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM) complexes [158]. Several proteases participates in the processing and degradation of PINK1 in the IMM: the mitochondrial processing peptidase (MPP) [159], AFG3L2 (a subunit of mitochondrial AAA protease in human) [160] and Rhomboid-7/presenilin-associated rhomboid-like protease (Rho-7/PARL) [161]. Processing by these proteases have been shown to release PINK1 in the cytosol where it is degraded by the proteasome [162]. An alternative route for PINK1 degradation after processing by these

three proteases could happen in the mitochondrial matrix where it is further broken down by the protease Lon [163]. Upon decrease of mitochondrial potential due to mitochondrial damages, import of PINK1 through the TOM complex becomes impossible. PINK1 is therefore stabilized on the OMM and activated via auto-phosphorylation. Once activated, PINK1 phosphorylates ubiquitin and the UBL domain of PARKIN, leading in turn to interaction of activated PARKIN with phospho-ubiquitin and translocation of PARKIN to the surface of the mitochondria. A feedforward mechanism is thus created, where PINK1 lifts the auto-inhibition of PARKIN and phosphorylates ubiquitin, leading PARKIN to build polyubiquitin chains on the surface of mitochondria, which will then be phosphorylated by PINK1, resulting in recruitment and activation of more PARKIN. Furthermore, PARKIN might help stabilizing PINK1 [164], thus creating a positive feedback loop. It is unclear however, if stabilization of PINK1 by PARKIN is dependent on PARKIN ubiquitination activity [165]. PARKIN has been shown to conjugate K6, K11, K63 [166] and even K48 [167] chains on damaged mitochondria as fast as 1 hour after loss of mitochondrial potential, in a ligation process dependent on its catalytic cysteine and phosphorylation by PINK1 on its UBL domain. This increase of ubiquitination by PARKIN has been quantified as a 6-fold increase 1 hour after depolarization of mitochondria [167]. The DUB USP30 acts as a regulator of mitophagy by counteracting PARKIN ubiquitination of mitochondria and this despite phosphorylation of ubiquitin chains [168]. *In vitro*, USP30 has been shown to have a strong preference for K6 and K11 ubiquitin chains on the OMM [166]. *In vivo*, USP30 seems to be much more promiscuous and removes all ubiquitin chains conjugated by PARKIN [167]. Other DUBs have also been described as regulator of PARKIN-dependent mitophagy, such as USP8 [62], USP15 [169] and Ataxin-3 [170]. Upon ubiquitination by PARKIN, autophagy adaptors are recruited to depolarized mitochondria: mostly SQSTM1/p62 [171] and NBR1 [172], although TAX1BP1 and NDP52 have been found as interactors of PARKIN [173]. Moreover, PARKIN overexpression recruits also OPTN to damaged mitochondria [174]. The LC3-interacting region (LIR) motifs of these autophagy adaptors then recruit LC3 bound to the omegasome/autophagosome, ultimately leading to degradation of the defective mitochondria in lysosomes. Among the mitochondrial proteins polyubiquitinated by PARKIN are Mitofusin (MFN) 1/2 and mitochondrial Rho GTPase (RHOT) 1/2 [173]. These GTPases mediate mitochondrial clustering and fusion in the case of MFN and mitochondria trafficking in the case of RHOT0. Their polyubiquitination results in separation of the mitochondria from the cytoskeleton (stopping their axonal transport in neurons for example), fragmentation of the mitochondrial network [175] and separation from the ER before undergoing degradation [176]. However, this idea of separation of mitochondria from the ER during mitophagy would go against previous studies indicating that contact sites between the mitochondria and the ER are crucial for autophagosome formation [177], unless these events are sequential: first autophagosomes are recruited, then loss of contact between ER and mitochondria occurs. Regarding RHOT, phosphorylation on its residues T298 and

T299 inhibits ubiquitination by PARKIN and mitochondrial arrest, indicating that mitophagy could be halted via the phosphorylation state of certain OMM proteins [175]. Other proteins from the OMM have been found as candidates for ubiquitination by PARKIN, such as TOM20, TOM70 and fission proteins (FIS1 and TBC1D15) [173]. Interestingly, the same study by Sarraf *et al.* indicates that PARKIN interacts with proteasome subunits (PSMC1, 2, 3, 5 and PSMD 8 and 13) and proteins involved in proteasomal degradation such as VCP/p97 [173]. This finding corroborates previous observations of PARKIN interacting with the proteasome [178] and being involved in recruitment of the UPS for degrading OMM proteins such as TOM20 and TOM70 [172,179], prior to removal of the whole mitochondrion by mitophagy. Another feedforward loop running downstream of PINK1 and PARKIN activation involves TBK1, a serine/threonine kinase phosphorylating OPTN, NDP52 and p62 [180–182]. Phosphorylation of these autophagy adaptors by TBK1 increases their affinity towards ubiquitin chains, which promotes mitophagy.

1.8.2.3 PARKIN-independent mitophagy

There is growing evidence that PARKIN is not the only E3 involved in mitophagy. Gp78 [183], SMURF1 [184], SIAH-1 [185] and ARIH1 [186] have all been found to regulate mitophagy independently of PARKIN but in a PINK1-dependent manner, by ubiquitinating mitochondrial substrates and recruiting autophagy adaptors such as OPTN, NDP52 and SQSTM1/p62 [187]. Although these E3 do not need PARKIN to induce mitophagy, they function in a similar way as PARKIN, requiring the activation of PINK1 upstream of their own activation, consequently activating the PINK1 feedforward loop.

1.9 TARGETED UBIQUITINATION

Since ubiquitination is a key post-translational modification for protein turnover and trafficking, it should come as no surprise that several targeted ubiquitination systems have been engineered to harness protein degradation in order to target proteins of clinical interest for degradation. Among such approaches are proteolysis targeting chimeras and ubiquibodies.

1.9.1 PROTACs

Proteolysis targeting chimeras (PROTACs) [188,189] are the most successful approach aiming at targeting specific proteins for degradation. PROTACs are able to bind a specific E3 ligase and the target protein, thereby bringing both proteins close enough for the target to be ubiquitinated. A PROTAC is a ligand for the target to be degraded, fused to a linker and a ligand for an E3 [190]. Most PROTACs are based on a hypoxia-inducible factor-1 alpha (HIF1α) moiety, which can recruit the von Hippel-Lindau (VHL)-cullin-RING ligase, leading to ubiquitination and proteasomal degradation of the target. PROTACs are based on a peptide scaffold and therefore of larger size than most small molecules. The HIF1α moiety, however, has been brought down to the minimal molecular weight of 400 Da by conserving the hydroxyproline moiety necessary to VHL binding, allowing better tissue distribution of the PROTAC [191].

1.9.2 Ubiquibodies

Another more recent approach to target proteins for degradation is the usage of ubiquibodies [192]. The idea behind ubiquibodies is to exploit the E3 CHIP (carboxyl terminus of Hsc70-interacting protein) to target a specific protein for degradation by replacing the binding domain of CHIP by an intrabody or monobody specific of the target. This technique is more generic than PROTACs because it does not require endogenous E3 and a designed ligand to bind both the target and the E3. The molecular weight of ubiquibodies is also larger than the one of PROTACs, making it less suitable in a therapeutic setting.

1.9.3 Limitations of current targeted ubiquitination system

One major limitation of both approaches is their focus on inducing proteasomal degradation of potential therapeutic targets. As it has been shown in previous parts of this introduction,

many types of ubiquitin chain topographies exist, and they do not always target for proteasomal degradation. Although targeting specific proteins of therapeutic interest is clinically relevant, these approaches cannot be used to deepen our understanding of the function of various ubiquitin-linkages. This is especially the case of PROTAC, which uses endogenous E3 to degrade an endogenous substrate, making it difficult to interpret results. Furthermore, the ubiquibody would still target an endogenous substrate, which is still subject to many different regulations in the cell. By targeting specific endogenous substrates, these techniques also have the caveat that off-target effects may occur. Therefore, one system that would clarify the role of specific ubiquitin chains in cells should be independent from endogenous regulations as possible and as such, no ubiquitination of the target by other unknown E3s should happen in order to make interpretation of results clearer. It is also desirable that the system is modular and easy to modify in order to generate different ubiquitin chain types.

2 AIM OF THE STUDY

The aim of this study was to develop a ubiquitination system where an engineered ubiquitin ligase can assemble K63 ubiquitin chains on a fluorescent substrate when brought in close proximity to a substrate-of-interest. Subsequently, we wanted to exploit this system to generate ubiquitin chains on the surface of mitochondria and investigate mitochondrial clustering and mitophagy (Paper I). Secondly, this work investigated another ubiquitin-dependent proteolysis in the nucleus in the context of the Hutchinson-Gilford progeria syndrome (HGPS). We aimed at verifying if the ubiquitin-proteasome system was impaired in human cells expressing a mtated version of lamin A, progerin (Paper II).

3 METHODOLOGICAL CONSIDERATIONS

3.1 HELA CELLS STABLY EXPRESSING SOLUBLE AND MITOCHONDRIAL GFP SUBSTRATE (PAPER I)

HeLa is a human cervical cancer cell-line from Henrietta Lacks, which is one of the most common human cell lines used in cell biology. HeLa cells are cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum. To generate HeLa cells stably expressing GFP-DmrA and mito-GFP-DmrA (see paper I), we started by transfecting our pCMV-IRES-puro-GFP-DmrA or mito-GFP-DmrA plasmid into HeLa cells. The transfection reagent used was Lipofectamine® 2000 (Invitrogen), which forms liposomes encapsulating the plasmid DNA and allow it to cross the cell membrane. Transfection is carried out in a T25 flask with Dulbecco's modified Eagle's medium devoid of fetal calf serum, because serum can interfere with interactions between the DNA-liposome complexes and the cell membrane, thus decreasing transfection efficiency. Forty-eight hours after transfection, selective pressure was applied to the cells by adding 1 µg/mL of Puromycin to the cell culture medium in order to select cells that integrated the plasmid into their genome. The 1 µg/mL dose had been determined as the minimum dose killing 100% of the HeLa cells after 5 days. The selection process goes on for 10 days during which the culture medium with 10% serum and 1 µg/mL puromycin is changed every third day. After these 10 days, the cells are detached from the surface of the flask using trypsin/EDTA and diluted to a concentration of 1 cell/mL. These cells were then plated on several 24-well plates, 500 µL per well (0.5 cells per well on average). After two weeks of culture in medium with puromycin, the expanded stable monoclonal mito-GFP-DmrA cells displaying moderate GFP fluorescence under an epifluorescence microscope were frozen.

One clone was further expanded and sorted using flow cytometry. Flow cytometry is a common laser-based method, allowing for sorting of single cells based on their fluorescence (either due to expression of a fluorescent protein or fluorescent antibodies), size and granularity. One cell at a time is passing through a capillary and is exposed to a set of lasers. In our case, GFP fluorescence was measured together with the granularity of the cell using sideward scatter (scattering of light around the cell at a 90° angle) and the size of the cell using forward scatter (scattering of light around along the path of the laser). This sorting allowed us to select HeLa cells exhibiting moderate GFP fluorescence because we anticipated that high expression of mito-GFP-DmrA might be detrimental to cellular growth and fitness of the cells.

3.2 CONFOCAL LASER SCANNING MICROSCOPY (PAPER I AND II)

Confocal laser scanning microscopy is a laser-based type of fluorescence microscopy using a pinhole to obtain high-resolution images, especially along the z-axis, by detecting light coming from the focal plane only. The light coming from planes out of focus does not pass through the pinhole and therefore does not reach the detectors. The detectors used in most confocal microscopes are photomutiplier tubes (PMT). These PMTs convert photons into an amplified electric (analog) signal, which is converted next into a digital signal, resulting in pixel intensities *i.e.* an image. Multiple fluorophores *i.e.* "colors" can be detected by using of an acousto-optic tunable filter (AOTF) and choosing carefully a set of fluorophores for the specimen (a recombinant fluorescent protein or coupled to antibodies) a set of lasers and PMTs. In both papers, images were acquired using a LSM510 Meta (Carl Zeiss) confocal laser scanning microscope with Plan Apochromat 63x/1.40 DIC oil immersion objective (Carl Zeiss), with a resolution of 1024 x 1024 pixel. For paper I, nuclei were counterstained using Hoechst 33342, which binds DNA and has a maximum excitation peak at 361 nm, while emitting a blue fluorescence at a maximum of 486 nm. The EGFP (Enhanced Green Fluorescent Protein) recombinant proteins in both papers have a maximum excitation peak at 488 nm and a maximum emission peak at 509 nm (green). The mCherry used in paper I has a maximum excitation peak at 587 nm and a maximum emission peak at 610 nm (red). HcRed1 used in paper II has a slightly different spectrum from mCherry: its maximum excitation peak is at 588 nm and its maximum emission peak is at 618 nm (far red). The cells were fixed in 4% paraformaldehyde prior to mounting on glass slides in Mowiol mounting medium. Using such a mounting medium is important for optimum imaging by avoiding a mismatch of the refraction index of the immersion medium (oil) with the mounting medium, avoiding autofluorescence and reducing photobleaching of the fluorophores.

3.3 WESTERN BLOT (PAPER I)

Western blot, also known as immunoblot, is a common molecular biology method for detection of proteins by using antibodies. One limitation of western blot is therefore the quality and performances of antibodies specific for the proteins-of-interest. It is also important to keep in mind that western blots are only semi-quantitative, meaning that it can give us ratios that describe differences and fluctuation of a protein levels in different conditions but not give a precise quantity of proteins in a sample. Samples can consist of purified proteins-of-interest or cell lysate. Sample preparation is followed by separation of proteins by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). SDS is used to denature proteins (together with boiling the sample) and charge them negatively. Negatively charged proteins

will then follow an electric field during the electrophoresis and migrate towards the bottom of the gel, where is located the positive pole of the system. The distance traveled by proteins depends on their mass and the density of the polyacrylamide gel, not on the proteins' charge, as all proteins are similarly denatured and charged by SDS. The mass of proteins can be estimated when compared to a molecular marker of known weight. Proteins are then transferred from the polyacrylamide gel to a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF). The transfer is followed by a blocking step. The aim of the blocking step is to reduce non-specific binding of antibodies to the membrane. Different type of blocking solutions can be used depending on the antibodies, such as nonfat milk powder or bovine serum albumin in phosphate-buffer saline (PBS). Then, the membrane is incubated in a primary antibody solution. The primary antibody is specific of the protein of interest and can be from monoclonal or polyclonal origin. Monoclonal antibodies are specific for an epitope on the protein-of-interest and polyclonal antibodies can recognize multiple epitopes, which might confer them a higher sensitivity, although binding to another protein beside the one of interest is more likely. After each incubation with an antibody, it is important to wash the membrane with PBS or TBS (depending on the antibody), to reduce non-specific binding of the antibody. The membrane is then incubated in a secondary antibody solution. The secondary antibody is coupled with Horseradish Peroxidase (HRP) and is specific of the primary antibody, which amplifies the signal. A final detection step is carried out, during which a substrate for the HRP is added to the membrane. Oxidation of the substrate by HRP ensues, leading to light emission, which is then enhanced by various chemicals in the detection reagent. The emission of light and its subsequent amplification is known as Enhanced Chemiluminescence (ECL).

3.4 IMMUNOPRECIPITATION (PAPER I)

Immunoprecipitation is commonly used in molecular biology to study the interaction between proteins, post-translational modifications of a protein of interest or purification of proteins. During an immunoprecipitation, cells are first lysed on ice, either in a denaturing buffer abolishing protein/protein interaction or a native buffer preserving such interactions. In our case, the native buffer is made of 10 mM Tris/Cl pH 7.5; 150 mM NaCl; 0.5 mM EDTA and 0.5% NP-40 while the denaturing buffer does not contain NP-40 but instead 1% SDS. The difference is that NP-40 is a mild nonionic detergent, sufficient to open cells and solubilize membrane proteins, while preserving protein/protein interaction and keeping proteins in their native conformation. SDS on the other hand, is a strong ionic detergent known for denaturing proteins, thus reducing protein/protein interaction. Soluble and insoluble proteins are separated by centrifugation, the soluble proteins are then incubated with antibodies coupled to beads, in our case: GFP-Trap® _A or RFP-Trap® _A (ChromoTek GmbH, Germany), which

are made of cross-linked 4% agarose beads coupled with anti-GFP or anti-RFP monoclonal antibodies from alpaca (*Vicugna pacos*). The beads are then spun down and boiled at 95°C into 2X SDS sample buffer (125 mM Tris/Cl pH 6.8, 20 % glycerol, 4 % SDS, 10 mM DTT and 0.02 % bromophenol blue) for 10 minutes to detach the pulled-down proteins from the beads. An immunoprecipitation is usually followed by a western blot in order to detect proteins and/or post-translational modifications.

3.5 PROTEIN PURIFICATION AND *IN VITRO* UBIQUITINATION ASSAY (PAPER I)

In vitro ubiquitination assays are used for characterizing ubiquitination enzymes (E1, E2 and/or E3) and their substrates in a simple and minimalistic system. In paper I, we used ubiquitination assays to study the functionality of the engineered E3 ligase ProxE3 and the type of ubiquitin linkages assembled on the substrate of ProxE3. Purified proteins are necessary for ubiquitination assays and can either be bought from a provider or purified. ProxE3, its GFP substrates and untagged ubiquitin mutants were purified from *E. coli* bacteria (BL21[DE3] for the fluorescent proteins and Rosetta[DE3]pLys for the ubiquitin mutants). The bacteria are first lysed, followed by immunoprecipitation (see above) for the purification of ProxE3 and its GFP substrate because these proteins are “tagged” with fluorescent proteins, which were used for immunoprecipitating the recombinant proteins. For untagged ubiquitin mutants, pH of the cell lysate is brought down to 4.5, which precipitates many proteins but not ubiquitin. The proteins that remain in suspension, including ubiquitin, are then concentrated by dialysis followed by spinning in 3.0 kDa Molecular Weight Cut-Off centricon tubes. Untagged ubiquitin mutants that are purified by this procedure are of sufficient in purity for *in vitro* ubiquitination assays while not being of the highest purity.

The ubiquitination assay itself was carried out in a ubiquitination buffer (25 mM Tris-HCl pH 7.6, 5mM MgCl2, 100 mM NaCl, 2 µM DTT) at 37°C with 0.2 µM of ProxE3/ProxE3* and GFP substrate and 0.5 µM AP21967 dimerizer. This first step allows dimerization of ProxE3 with its GFP substrate. Then, the ubiquitylation reaction was started by adding 200 nM UbE1, 1 µM UbcH5C, 1.2 µM untagged or HA tagged (Boston biochem) ubiquitin and 2 mM ATP to the pre-dimerization mix. The reaction was stopped by adding one volume of 2x SDS-PAGE sample buffer and boiling at 95°C for 10 minutes, followed by western blot (see above).

4 RESULTS AND DISCUSSION

4.1 PAPER I

The first paper aimed at developing and testing a molecular tool, ProxE3, for investigating the role of K63 polyubiquitination in specific organelles. The ProxE3 recombinant protein has been generated from the fusion of a FRB dimerization domain at the N-terminus, a NEDD4-1 HECT domain in the C-terminus and a mCherry fluorescent protein in the middle. The target of ProxE3 is a GFP recombinant protein with GFP at the N-terminus, fused with a FKBP12 dimerization domain in the center and a linker containing 5 lysines in the C-terminus. This GFP substrate is largely inherent in mammalian cells and is therefore ideal to study the effect of a specific ubiquitin linkage on the fate of a protein. Upon treatment with the rapalog AP21967, the FRB and FKBP12 domains interact, bringing ProxE3 in close proximity of its GFP substrate. We have shown that it is possible to conjugate specifically K63-linked polyubiquitin on the GFP target in HeLa cells expressing both proteins and *in vitro*. Such ubiquitination of the GFP substrate does not lead to its degradation, be it by the proteasome or autophagy.

Interestingly, ProxE3 was able to auto-ubiquitinate both *in vitro* and in cells, leading to its proteasomal degradation in the latter, as proteasomal inhibitor MG132 stabilizes ProxE3. This is in line with the literature indicating that the HECT domain from the NEDD4 family tend to auto-ubiquitinate in absence of a substrate when it is expressed without the regulatory WW or C2 domains. However, since ProxE3 conjugates K63 ubiquitin linkages, it is uncertain how it can be degraded by the proteasome. It is possible that K63-linked ubiquitin chains conjugated by ProxE3 on itself can be edited by other E3s, as it is known to happen to Mdm2, GP78 and CBL proteins [193]. Also, the HECT E3 HUWE1 has been shown to branch K63 ubiquitin chains with K48-linkages [194], although this has not been shown for any endogenous E3s. Proteins such as A20 could also be candidates. A20 is known to bind K63-linked ubiquitin chains and has a DUB activity towards this linkage. However, it also has a K48 ligase activity [195]. This allows A20 to remove K63-linked ubiquitin chains from its target, RIP1, and replace them by K48-linked ubiquitin, leading to RIP1 proteasomal degradation [196].

The GFP substrate was expressed in a specific organelle: the mitochondrion, by adding a mitochondrial transmembrane domain in C-terminus. ProxE3 was also able to conjugate K63-linked polyubiquitin on the mitochondrial GFP substrate. Although it did not lead to mitophagy, even after depolarization of mitochondria with CCCP, clustering of mitochondria was observed. The absence of mitophagy despite the presence of K63-linked ubiquitin chains on

the GFP mitochondrial substrate in depolarizing conditions could be explained by an insufficient amount of K63 ubiquitin on the mitochondria to trigger mitophagy. This amount of K63 ubiquitination might be enough to trigger peri-nuclear clustering of mitochondria in our artificial system however. Another related explanation would be that the PINK1 feedforward loop is insufficient in our system. Even in PARKIN independent pathways, the PINK1 feedforward loop is required to recruit more E3 ligases and phosphor-ubiquitin to the mitochondria. However, ProxE3 being an exogenous recombinant protein, it is not further recruited by the PINK1 feedforward loop and functional PARKIN might be present at a too low level in HeLa cells to sustain mitophagy signals [197,198]. It could also be that other types of ubiquitin chains are required to trigger mitophagy. In fact, PARKIN is known to conjugate K48, K6 and K11 linkages on top of K63 linkages, while our ProxE3 conjugates only K63-linked polyubiquitin. Finally, ubiquitination of a specific mitochondrial protein might be necessary to trigger mitophagy.

The novelty of this work is a new modular tool for studying the role of specific ubiquitin linkages on organelles, without the need of targeting a specific endogenous substrate. This paper also reaffirms that E3s are tightly regulated and can potentially be targeted by other E3 or ubiquitin editing proteins. We also give evidence that an increase of K63 chains on a mitochondrial substrate is not enough in itself to trigger mitophagy but can trigger clustering of dysfunctional mitochondria.

4.2 PAPER II

Paper II focused on HGPS, a rare genetic disorder leading to premature aging in children. The model developed in this study is an inducible transgenic mouse model expressing the most common human lamin A mutation responsible of HGPS, LMNA c.1824C>T. The resulting nuclear protein, which is commonly referred to as *progerin*, is a truncated pre-lamin A lacking 50 amino acids. This work focused primarily on potential effects of progerin in the brain of HGPS mice, although no detrimental effects were observed in the brain and behavior of animals even after 90 weeks of expression of the transgene. Nonetheless, tissue specific accumulation of progerin has been observed in neurons of the frontal cortex and hippocampus of mice expressing the transgene for 90 weeks compared to those expressing it for 20 weeks. Furthermore, severe distortion of the nucleus (multiple lobulations and irregular extensions) of hippocampal neurons has been observed in transgenic mice compared to wild-type. These observations led to investigating if the ubiquitin-proteasome system was compromised in cells of transgenic mice, due to accumulation of progerin. To explore the potential impairment of the UPS due to progerin overexpression, a human melanoma cell-line (MelJuSo) stably expressing a GFP reporter substrate of the UPS was used. No accumulation of the GFP reporter substrate was observed in the stable cell line overexpressing progerin compared to those overexpressing lamin A. In comparison, treatment of these cells with MG132, a proteasome inhibitor, lead to accumulation of the GFP substrate. These experiments suggested that the UPS remains functional, even when progerin is overexpressed, which contradicts previous studies reporting impairment of the catalytic activity of the proteasome by progerin in fibroblasts of HGPS patients [199]. The lack of ubiquitin-rich lamin A, progerin or even Tau inclusions in cortical and hippocampal neurons of 90-week HGPS mice compared to WT, further support the hypothesis of an absence of UPS impairment in HGPS mice. The novelty of this paper is to imply that there is no global impairment of the UPS in cells expressing progerin.

5 CONCLUDING REMARKS

Overall, this work shows how tightly regulated and robust ubiquitin-dependent cellular processes are. On one hand, expression of the mutated form of a major component of the nuclear envelope, lamin A/progerin, does not affect the capacity of the UPS to degrade its target proteins, despite the accumulation of this mutated protein in the nucleus under pathological conditions. On the other hand, adding K63-linked ubiquitin chains on the surface of mitochondria is insufficient to trigger mitophagy, even after depolarization of mitochondria. In both cases, lack of robustness and regulation of these pathways would lead to cell death: by proteotoxic stress in the first case and by rapid degradation of the mitochondrial network in the second case.

Although K63 ubiquitination is necessary for mitophagy, it is not sufficient. Commitment to the mitophagy route might require more ubiquitin chains, different type of ubiquitin chains or further activation of PINK1 and amplification of the signal. Despite the lack of mitophagy triggered by the ProxE3 system, this tool can be used for conjugation of specific ubiquitin chains on an exogenous substrate in cells. The present work has only used this tool in the context of mitophagy but it could be of use in different cellular contexts, such as for studying ubiquitination of integral inner nuclear membrane proteins. The degradation of integral inner nuclear membrane proteins remains elusive and has mostly been studied in yeast so far [81,82]. The ProxE3 tool could also be used to study the impact of specific ubiquitin-linkages on protein aggregates.

6 ACKNOWLEDGEMENTS

I would first like to thank my supervisor, **Nico Dantuma**, for the opportunity to start in his group but also for his support and understanding during the past few years, despite all the sweat and tears (no blood though… luckily!). I have learned a lot!

I also want to thank my labmates from the Dantuma lab, past and present, from whom I have learned a lot (**Florian**, **Daniela**, **Laura Bott** and **Klara**) and who managed to turn tough times into fun times (**Tatiana**, **Laura Herzog**, **Annika**, **Melania** and **Francesco**). I wouldn't have made it without you!

I want to thank my co-supervisor, **Olle Sangfelt**, and my mentor, **Arne Lindqvist**, for their advice and support.

A big thank you to **Aldwin**, for the biological tips and tricks but also for the nice conversations about science but also life in general. Thanks for believing in me and telling me to hang in there!

I am also thankful to the first bunch of people I have met, here in Stockholm and which helped me going through the first year of my studies: the **Jägargatan family**. They are too numerous to name but I will try anyway! Thanks to **Frank, Michele, Vania, Antero, Jonas, Pradeep, Nandy, Lizan, Michael (Chrobok), Gunnhildur, Triin, Robin, Ioana, Aline, Alkesandra, Oliver, Ferdi, Karen, Kilou, Nicolas/Jean-Claude** and **Hoyin**. You were/are all amazing!

I want to thank the CMB Pub Crew (**Helena**, **Goncalo**, **Isabelle**, **Yildiz**, **Giuseppe**, **Michael Gralla**, **Jeff**, **Millind**, **Pedro R.** and **Pedro V.**) for all these fun late nights.

I am also thankful to other cool CMB people: **Mehdi**, **Milos**, **Micke**, **Jens**, **Him**, **Mauricio**, **Alberto**, **Gianvito**, **Erik**, **Ivanna**, **Francesca** and **the Benjamins** (Braune and Ka-Cheuk Liu). Thank you all for the chats at the coffee machine, during retreats or in the corridors between experiments!

Julian, thanks for being an amazing friend in my early days in Stockholm and kicking my butt when I was feeling down because of the weather and darkness. You were truly the beating heart of the "Jägargatan family". Keep on being intense and embracing life!

Szabi, thank you for being a great roommate and friend. We shared so many great (and also downright scary) moments together! I will always cherish these memories and your friendship.

Aga, thanks for being an amazing roommate which decorated the apartment with great taste. We did not always hang out as much as I would have liked but know that I see you as a great and reliable friend, forever.

Mat, thanks for sharing part of your life with me. We've had some amazing/crazy parties but also some tough times that we managed to overcome. You've helped me in many ways.

Pim and **Jolinde**, you are both amazing and I am proud of counting you among my best friends! You have always been so supportive of me, I just don't know what to say or how to thank you besides "you will always be welcome under my roof and in my heart".

Shahul, we have shared so much! I will always remember vividly the first time we have met at CMB pub and your introduction seminar. You are an amazing person and I hope we will still be friends, even in a hundred years, telling tall tales, by a cozy fire place, surrounded by our grand-children!

Tiago, you are Dr. Awesome! You have been so supportive and helpful, I feel so grateful every time I think about it! I am proud to count you among my best friends. What to say? Just a huge heartfelt "Thank you" I guess!

Niko, we have been through good and bad together but we always have had each other's back. I see you as a soul brother and I am overwhelmed with joy and pride at this realization.

Lisa, thank you for being an amazing person! I have to admit (and I am slightly ashamed of it), the first time I have met you I thought you were way too nice to be real! But you are THAT nice! I wish you the best across the pond with Jens and your baby boy. Puss och kram. ☺

Michael (Hagemann-Jensen), thank you for all the fun and good times at KI but also in Copenhagen and for being an overall great friend and support when needed!

Richard, thank you for being such a great friend! Know that you will always be welcome, wherever I live. To think we did all that and may I say, not in a shy way oh no, no, not me, we did it our way!

Björn, thank you so much for being one of my best friend, here in Sweden! You have such a big heart and you are such a great human being! We have shared plenty of times and experiences together but I am sure it is just the beginning!

Christina Mannerheim, thank you for your hospitality, your support and your friendship! I would have never skied and all our projects during the master would have failed without you. If you ever want to stay in a nice place in the heart of Bretagne, just give me a call!

Eliott, **Becky**, **Dara** and **Wolfgang**, thank you my friends for showing me how lame "lagom" was. Thank you for all the great memories and your friendship.

Shane, **Thibault**, **John** and **Jonathan**, merci pour tout les gars! Vous avez été de super potes pendant toutes ces années et j'espère qu'on se reverra à l'occasion, que ce soit en Suède, en France, en Suisse ou au Canada!

Karen and **Juan**. Wow! What to say ? You are both amazing! Know that I value your friendship and your support very much and wherever life brings us, I want to remain friends with you until my very last breath!

Ineke, thanks for being a great friend! Thanks for the great conversations, the fun times and the adventures in hostile environments (no, I am not talking about academia).

Patrick and **Uta**, thank you for EVERYTHING! From the good times during midsummer, to BBQs, parties, gaming nights, the moral support during tough times. Just, thank you! There will be more for sure!

Anthony, thank you mate! For being an amazing friend and human being! For making me laugh and smile even when I thought I couldn't and for connecting me with so many amazing people.

Marijke, thanks for the great conversations and your contagious smile and laugh! You are a ray of sunshine.

Helena Storvall, thank you for the fun talks and the great board game sessions. I hope there will be many more!

JP, thank you for being a great friend! You always manage to cheer me up. And I love our crazy talks about science, even late at night on a week-end. Keep on being awesome!

Rebecca, thank you for your support during my endeavor, here in Sweden. I have learned so much about Sweden but also about myself at your contact. Thank you for everything and I am sure we will remain good friends!

Kim, thank you for your infectious laugh and happiness! I will never forget the great times we have had in Copenhagen. I have not assembled the birthday present you gave me years ago. It is still there, throning on my bookshelf in its box, as a reminder of how much good friends can cheer you up on a tough day.

David and **George**, thanks for being cool people! We have not hanged out together as much as I would have liked but I really enjoyed the few hours we have spent together and I wish there will be more!

Veronika and **Theresa**, thank you for the cool conversations about science, career and life in general!

Nevin, thanks for all the laughs and passionate talks!

Ylva, thanks for all the Marvel nerdiness and the amazing mead tasting. These were good times! Let's watch the next Avengers together!

Ahmed, thank you for the great conversations about science but also our personal lives. It really helped me going forward. You will always be welcome in Stockholm for as long as I am living there!

Rubin, thanks for all the fun and crazy times!

Francesca Bignami, thank you for all the great conversations and for just being there at these nice, chill dinners at Shahul's!

Anneliese, thank you for being such a cool person and all the crazy work you put on the 4th of July every year! It is always a highlight of my year, every year. Thank you!

Mirko, I know you want to be the very best like no one ever was! It's you and me, I know it's our destiny!

Per, thanks for being a great colleague and friend. We have learned so much together and I wonder where our next adventure will take us!

Claudia, thank you so much for helping me with this work at Tobii! It really helped me. Know that you will always have my utmost respect and that I value our friendship.

My esteemed fellows of the Marie Curie network: **Lara**, **Vicki**, **Irene**, **Anna**, **Raffaella**, **Metin**, **Matthias**, **Raheleh**, **Gemma**, **Soumya**, **Geetha** and **Mariana**. So many adventures and good times together!

Achraf et tout le Discord MH pour m'avoir soutenu durant les heures sombres de mon histoire.

Merci à tout mes amis de France : **Thomas**, **Florent**, **FX**, **Etienne**, **Geoffroy**, **Ludo**, **David, Yoann** et **Axel**. Vous avez tous été incroyables durant toutes ces années et j'adore vous retrouver une à deux fois par an sur Rennes (ainsi qu'en ligne, évidemment).

Un énorme merci à ma famille adorée ! Merci papa et maman d'avoir été mes plus gros supporters et d'avoir toujours crus en moi. Il va sans dire que je n'aurai jamais réussi sans avoir des parents comme vous !

… And last but not least, mon frère, **Gautier**. Tu es la personne la plus chère à mon cœur et je suis fier de toi ! \[T]/

Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081).

Illustrations for figures were produced using InkScape (https://inkscape.org/en/), under GNU General Public License.

This work has received funding from the European Community's Seventh Framework Programme FP7/2010 under grant agreement no. 264508.

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